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Eicosapentaenoic acid suppresses angiogenesis via reducing secretion of IL-6 and VEGF from colon cancer-associated fibroblasts

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Abstract. Eicosapentaenoic acid (EPA) improves interleukin (IL)-6 hypercytokinemia in patients with advanced cancer due to its anti-inflammatory effects. This EPA mechanism has been revealed to lead to several anticancer effects. While the effects of EPA on cancer cells have been investigated, particularly in terms of angiogenesis, its effects on the tumor stroma remain unclear. In the present study, the authors clarified the role of EPA in cancer angiogenesis against colon cancer-associated fibroblasts (CAFs) from the colon stroma. With established human CAFs and normal fibroblasts from colon stroma (NFs), the authors evaluated IL-6 and vascular endothelial growth factor (VEGF) secretion with or without EPA treatment using ELISA. The signal inhibition of mitogen-activated protein kinase (ERK) in CAFs by EPA was evaluated using western blotting. *In vitro* anti-angiogenesis effects were evaluated by the angiogenesis assay on Matrigel using human umbilical vein endothelial cells (HUVECs) cultured with the supernatant obtained from CAF cultures with or without EPA. IL-6 secretion was greater from CAFs compared with that from NFs and stimulation with lipopolysaccharide (LPS) resulted in greater IL-6 secretion from the two fibroblast types compared with that from fibroblasts without LPS stimulation. While LPS stimulation increased VEGF secretion from the two fibroblast types, EPA decreased IL-6 and VEGF secretion from CAFs. Western blotting revealed that the addition of 30 μM EPA inhibited the ERK phosphorylation signal in CAFs. Furthermore, the angiogenesis assay with Matrigel revealed that the CAF culture supernatants treated with EPA suppressed tubular formation in HUVECs. These reductions may have been caused by the inhibition of ERK phosphorylation by EPA. Thus, EPA reduces cancer angiogenesis associated with CAFs. Additional studies will be needed to clarify the continuous anti-angiogenetic effect of chemotherapy using angiogenesis inhibitors (e.g. bevacizumab and aflibercept) in conjunction with or without EPA, and the clinical usage of EPA in conjunction with chemotherapy *in vivo*.

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Abbreviations: EPA, eicosapentaenoic acid; VEGF, vascular endothelial growth factor; CAF, cancer-associated fibroblast; HUVECs, human umbilical vein endothelial cells; IL-6, interleukin-6; LPS, lipopolysaccharide; PUFA, polyunsaturated fatty acid; CSI, cancer-stromal interaction

Key words: eicosapentaenoic acid, vascular endothelial growth factor, cancer-associated fibroblast, human umbilical vein endothelial cells, anti-angiogenesis

Introduction

In this decade, the study of cancer microenvironments has garnered attention as it may help to elucidate novel anticancer mechanisms, which have not been exploited with the use of conventional cytotoxic anticancer drugs (1). Various mesenchymal cells observed in tumors have been hypothesized to serve passive roles in tumor growth (1). However, studies have revealed that these mesenchymal cells instead serve an active and irreplaceable role in cancer cell growth in terms of proliferation, invasion and angiogenesis (2,3). Angiogenesis is an essential mechanism for the metastasis and proliferation of cancer cells (4). Thus, anti-angiogenetic drugs, including bevacizumab and aflibercept, are regarded as important for colorectal cancer therapy (5). It has been hypothesized that angiogenesis in cancer is caused by cancer cells (5). However, recent studies revealed that stromal cells in cancer cause angiogenesis (6,7). Additional studies are needed to clarify the mechanism of angiogenesis caused by stromal cells. An increasing number of studies have focused on the interaction between tumor cells and stromal cells (cancer-stromal interaction, CSI), which serves an important role in the growth of several cancers (1,8,9). Among them, a previous study by our group demonstrated that inflammation causes VEGF secretion from cancer-associated fibroblasts (CAFs) in tumors via interleukin (IL)-6, which is secreted by CAFs and not by cancer cells (10). IL-6 may be the key cytokine that promotes VEGF

secretion from CAFs and thus VEGF levels may be decreased by a drug that decreases IL-6 levels.

Eicosapentaenoic acid (EPA), a polyunsaturated fatty acid (PUFA), is a well-known fish oil; EPA has been reported to decease systemic inflammation, which is caused by IL-6 (11,12). EPA has also been demonstrated to decrease inflammation caused by cancer (13). It has reported that EPA decreased IL-6 secretion in esophageal cancer cell lines (11). Nevertheless, a previous study by our group has revealed that a greater amount of IL-6 was released by cancer stromal cells than by cancer cells (10). At present, various mechanisms of EPA that effect cancer cells have been clarified (14-19). Certain PUFAs have also been revealed to inhibit VEGF expression in colon cancer cells (20,21). However, how EPA influences cancer stromal cells and the CSI has not yet been demonstrated. Thus, in the present study, the authors investigated the *in vitro* effects of EPA on cancer stromal cells, with regards to angiogenesis.

Materials and methods

Agents. Sodium salts of 5-, 8-, 11-, 14-, and 17-EPA were procured from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). EPA (5 mg) was added to 154 μl of 100% ethanol to prepare the stock solution, which was then diluted to yield a final ethanol concentration of 0.03%. Lipopolysaccharide (LPS, from *E. coli* O111:B4; 10 mg; Sigma-Aldrich; Merck KGaA) was concentrated to yield a final concentration of 50 μg/ml. Anti-IL-6 myeloma receptor antibodies (MRA) were kindly provided for free by Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). To certify the established cell lines as fibroblasts, anti-α-smooth muscle actin (α-SMA) monoclonal (1A4; cat. no. ab7817), anti-IL-6 (cat. no. ab9324) and anti-VEGF (cat. no. ab52917) antibodies were purchased from Abcam (Cambridge, MA, USA) and used for immunofluorescence staining. Additionally, anti-vimentin (V9; cat. no. M072529), anti-cytokeratin (AE1/AE3; cat. no. M351529; both Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) and anti-cluster of differentiation (CD) 90 (5E10; cat. no. BD-555593; BD Pharmingen; BD Biosciences, San Jose, CA, USA) mouse monoclonal antibodies were purchased. To confirm the effect of EPA on fibroblasts, p44/42 mitogen-activated protein kinase (MAPK, also known as ERK1/2 or ERK) monoclonal (cat. no. 4695) and phosphorylated ERK (Thr202/Tyr204; p-ERK; cat. no. 4377; both Cell Signaling Technology, Inc., Danvers, MA, USA) antibodies were used. Anti-GAPDH antibodies were purchased from Abcam (Cambridge, MA, USA; cat. no. ab9485). U0126, a dual specificity mitogen-activated protein kinase kinase 1 (MEK)/ERK inhibitor, was purchased from Cell Signaling Technology, Inc.

Isolation and culture of human colon fibroblasts. The authors of the present study established fibroblast cell lines from a specimen resected from a 73-year-old Japanese female patient with well-differentiated colon cancer at Department of Gastroenterological Surgery, Nagoya City University (Nagoya, Japan) in January 2016. The technical procedure was described in a previous report (22) and was performed as described previously (10). Following receiving written informed consent, tissues were retrieved from two separate regions: Colon carcinoma tissue and non-malignant colon tissue. To avoid contamination, these tissues were collected from the serosal side, taking special care not to pierce the mucosa. Following fragmenting with scissors, the tissue was incubated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA) containing 1,000 U/ml Dispase® (Godo Shusei Co., Ltd., Tokyo, Japan) for 2 h. Then, the fragments were cultivated in DMEM containing 5% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% antibiotic-antimycotic solution, and incubated at 37˚C in air containing 5% CO_2. To confirm that the cultivated cells were fibroblasts, the cells were examined using immunofluorescence to determine whether they expressed α-SMA, IL-6 and VEGF. Fibroblasts isolated from malignant regions or benign regions were termed as CAFs or normal fibroblasts (NFs), respectively. Fibroblasts in the third to sixth passage were used in subsequent experiments. Patient 1 was a 42-year-old Japanese male patient with well-differentiated colon cancer. Patient 2 was a 74-year-old Japanese male patient with poorly-differentiated colon cancer. The protocols used for sample collection and fibroblast examinations of Patient 1 and 2 were the same as described above.

Immunofluorescence staining. Immunofluorescence staining of the cultured cells was performed according to a method described previously (10). CAFs and NFs were grown in chamber slides, fixed with 10% formalin for 10 min at room temperature, treated with 0.2% Triton X-100 for 10 min at room temperature, and blocked with 1% bovine serum albumin (BSA; cat. no. WDH4409; Wako, Osaka, Japan) in PBS for 1 h at room temperature. Then, the slides were treated with anti-α-SMA (1:100) or anti-IL-6 (1:1,000) mouse monoclonal antibodies for 2 h at room temperature, and then with Alexa Fluor 488®-conjugated donkey anti-mouse immunoglobulin (Ig)G secondary antibodies (1:200; cat. no. ab15019; Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at room temperature. The membranes were also incubated with anti-VEGF rabbit monoclonal antibodies (1:500) for 2 h at room temperature and then with Alexa Fluor 488®-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. ab150077; Abcam) for 1 h at room temperature. These slides were mounted with ProLong Gold Antifade Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and observed under a BZ-X700 fluorescence microscope (Keyence Corporation, Osaka, Japan) equipped with a charge-coupled device camera. The magnification was x200.

Immunostaining. To confirm the phenotypic characterization of CAFs and NFs, anti-vimentin (V9), anti-cytokeratin (AE1/AE3) and anti-CD90 (5E10) mouse monoclonal antibodies were used as described by a previous study by our group (10). Briefly, CAFs and NFs were fixed with 10% formalin for 10 min at room temperature to cross-link the proteins, then blocked with 3% BSA in PBS for 1 h at room temperature. Resin was not used for this protocol. Then, the primary antibodies (vimentin, 1:80; CD90, 1:100; cytokeratin, 1:80) were applied for 60 min at room temperature. Horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. K4001; Dako; Agilent Technologies, Inc.) was applied for 60 min at room temperature. These slides were observed under a BX51 light microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x100.

Cell lines. Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell GmbH (Heidelberg, Germany). They were cultivated in Endothelial Cell Growth Medium (EGM) kit (PromoCell GmbH) and incubated at 37˚C in air containing 5% CO_2. EGM contained 2% fetal calf serum, 0.4% endothelial cell growth supplement, epidermal growth factor (0.1 ng/ml), basic fibroblast growth factor (1 ng/ml), heparin (90 μg/ml) and hydrocortisone (1 μg/ml; all PromoCell GmbH).

Cell viability assay. The viability assay was performed using Premix WST-1 Cell Viability Assay System (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Each assay was conducted at least three times (n=5). Fibroblasts ($3x10^3$/well) and HUVECs ($5x10^3$/well) were seeded into 96-well plates and incubated overnight. Next, the cells were treated with various concentrations (0, 10, 30, 50, 70 and 90 μM) of EPA in serum-free DMEM and incubated at 37˚C in air containing 5% CO_2. After 48 h, the medium was removed and replaced with fresh medium (90 μl/well) containing Premix WST-1 (10 μl/well). The cells were then incubated at 37˚C for 2 h. Absorbance was measured at 450 nm using a SpectraMax 340 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA, USA). The control cells were untreated.

Furthermore, WST-1 assays were conducted on the fibroblasts after 0, 24,48 and 72 h following the incubated with 0, 10 or 30 μM EPA. Absorbance was measured at 450 nm. The absorbance values of each EPA concentration was compared with the value at 0 h.

ELISA. Fibroblasts were seeded into 6-well plates at a density of $3x10^4$ cells/ml with 5% FBS in DMEM. Following overnight incubation, the fibroblasts were divided into four groups and cultured in serum-free DMEM; the groups were as follows: i) Untreated, ii) 30 μM EPA, iii) 50 μg/ml LPS, and iv) 50 μg/ml LPS and 30 μM EPA (9.7 μg/ml) Additionally, CAFs were divided into several groups and cultured in serum-free DMEM; the groups were as follows: i) Untreated, ii) 50 μg/ml LPS, iii) 50 μg/ml LPS and 10 μg/ml MRA (Actemra®; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan), iv) LPS (0, 0.1, 1, 10 or 100 μg/ml) with or without 30 μM EPA, v) IL-6 (0, 1 or 10 μg/ml; cat. no. 550071; BD Pharmingen; Franklin Lakes, NJ, USA) with or without 30 μM EPA, vi) 30 μM EPA, and vii) 10 μM U0126 (3.8 μg/ml). The untreated cells were incubated with concentrated 0.03% ethanol in serum-free DMEM and were used as the controls. After 48 h, the culture supernatants were collected and centrifuged (12 x g, 4˚C, 3 min) to pellet any detached cells. Their VEGF and IL-6 expression levels were quantified from the supernatants using VEGF (cat. no. DVE00) and IL-6 (cat. no. D6050) ELISA kits (both R&D Systems Inc., Minneapolis, MN, USA), respectively. The ELISAs were performed according to the manufacturer's protocol. All experiments were performed three times in triplicate. A lot of the *in vivo* cells possessed so few IL-6 receptors on their membranes; therefore the authors of the present study incubated these cells with soluble receptors. IL-6 was supplemented with soluble 40 ng/ml IL-6 receptor (cat. no. 200-26R; Human recombinant soluble IL-6 receptor was purchased from Peprotech; Rocky Hill, NJ, USA).

Western blotting. CAFs ($1.0x10^5$/ml) were seeded into 10-cm dishes with 5% FBS in DMEM and incubated at 37˚C in a CO_2. incubator until fibroblast growth was semi-confluent. After the cells were cultured in serum-free DMEM for 3 h for starvation, the medium was changed. The cells were cultured for an additional 3 h without treatment or in the presence of EPA (10 or 30 μM) or 10 μM U0126. Untreated cells were incubated with concentrated 0.03% ethanol in serum-free DMEM and were used as the controls.

Protein samples were extracted from CAFs using radioimmunoprecipitation lysis buffer with Protease Inhibitor Single Use Cocktail and Phosphatase Inhibitor Cocktail (all Thermo Fisher Scientific, Inc.), as described previously (23). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of the protein extract were denatured by boiling at 90˚C for 5 min. Proteins (20 μg/lane) were fractionated on 4-15% Mini-PROTEAN TGX gels and the protein bands were transferred onto nitrocellulose membranes (both Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primary and secondary antibody reactions were performed using an iBind Flex Western System (Thermo Fisher Scientific, Inc.). The membranes were incubated with iBind Flex Solution (iBind Flex Buffer, iBind Flex Additive and distilled water) for 10 min at room temperature to block nonspecific binding. The membranes were incubated with anti-ERK, -p-ERK (both 1:1,000) and -GAPDH (1:2,500) primary antibodies, and then HRP-conjugated goat anti-rabbit polyclonal secondary antibodies (1:2,000; cat. no. P0448; Dako; Agilent Technologies, Inc.). These primary antibodies and secondary antibodies reactions were performed at room temperature for 2.5 h. Protein-antibody complexes were visualized using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). The immunoreactive protein bands were detected and the band densities were quantified by densitometry using an Amersham Imager 600 with Amersham Imager 600 analysis software (version 1.2; GE Healthcare Life Sciences, Little Chalfont, UK). Experiments were performed at least three times.

Angiogenic effects of EPA. To study the angiogenic effect of EPA, the capillary-like structure formation (CSF) of the HUVECs was evaluated using the angiogenesis assay on Matrigel (BD Biosciences) with or 30 μM without EPA. For the reconstitution of a basement membrane matrix, Matrigel was diluted to 50% concentration with cold serum-free DMEM and added to a 24-well tissue culture plate (250 μl/well) at 4˚C. The 24-well plate was incubated for >2 h at 37˚C to allow the Matrigel to solidify, as described previously (24).

HUVECs were trypsinized, counted and resuspended in EGM, and added to the surface of the reconstructed basement membrane ($5x10^4$ cells/well). The medium of cultured CAFs were removed and termed conditioned medium (CM), which was diluted to 50% concentration with EGM. HUVECs were divided into three groups and cultured for 16 h to allow the CSF to take place; the groups were as follows: i) Serum-free DMEM and untreated, ii) serum-free DMEM and 30 μM EPA, iii) serum-free DMEM and 500 pg/ml VEGF (Recombinant VEGF165; cat. no. 890220; R&D Systems, Inc.), iv) CM and untreated, ii) CM and 30 μM EPA, iii) CM, 30 μM EPA and

500 pg/ml VEGF. These endotubes were quantified by counting nine fields (three regions per plate) at a magnification of x40, with each condition being assessed in triplicate. These were observed with inverted phase contrast fluorescence microscopy using a BZ-X700 fluorescence microscope. Untreated cells were incubated with concentrated 0.03% ethanol in serum-free DMEM and were used as the controls.

Statistical analysis. All statistical analyses were performed using EZR software (Easy R) version 1.27 (Saitama Medical Center, Jichi Medical University, Saitama, Japan). Data are presented as mean ± standard error of the mean. Differences between groups were compared by Student's t-test or a one-way analysis of variance followed by Tukey's test. P<0.05 indicated that the difference between groups was statistically significant.

Results

IL-6, VEGF and α-SMA expression is elevated in isolated CAFs. Cells with large cytoplasm and spindle-shaped cells were observed in samples from normal and cancerous colon stroma. The immunofluorescence staining revealed that the two cell types from colon stroma (CAFs and NFs) secreted α-SMA, IL-6 and VEGF (Fig. 1A). In addition, immunostaining demonstrated that CAFs and NFs were positive for expression of CD90 and vimentin, and negative for cytokeratin. Due to the high expression α-SMA and IL-6, the cells from human colon stroma were regarded as fibroblasts; as the cells from the colon cancer stroma exhibited a high expression of α-SMA, an indicator of CAFs (10), they were clarified as CAFs. It should be noted that CAFs have properties of myofibroblasts and express α-SMA (25). The expression level of IL-6 was higher in CAFs compared with that of NFs, whereas the expression of VEGF and α-SMA in NFs and CAFs was almost the same. IL-6 and VEGF secretions from CAFs and NFs were also evaluated using ELISA (Fig. 1B). The expression of IL-6 (P<0.001) and VEGF (P=0.001) cultured in 5% FBS in DMEM was significantly higher in CAFs compared with NFs; IL-6 and VEGF expression was 5.0- and 1.5-fold higher, respectively.

Human colon fibroblasts and HUVECs remain viable with 30 μM EPA treatment. The WST-1 assay demonstrated that EPA suppressed the viability of CAFs, NFs and HUVECs in what appeared to be a concentration-dependent manner (Fig 1C). At >50 μM EPA, fibroblast viability reached a plateau. However, the viability of CAFs, NFs and HUVECs were not suppressed by treatment with <30 μM EPA, with ~80% viability observed compared with the control samples. The viability of CAFs, NFs and HUVECs after treatment with 30 μM EPA was 95.1, 79.2 and 92.1%, respectively; no significant difference in the viability of these cells was identified compared with their respective controls. Furthermore, no significant decrease in the viability of CAFs and NFs was identified when the cells were incubated with 30 μM EPA for 24, 48 or 72 h (Fig. 1D). In NFs, it is still unknown why the viability increased with 10 μM EPA. Thus, in the present study, the authors used EPA at a concentration of 30 μM to evaluate the suppression of VEGF and IL-6 secretion without viability suppression.

EPA reduces VEGF and IL-6 secretion in NFs and CAFs with or without LPS-stimulation, and reduces IL-6-induced VEGF secretion in CAFs. ELISA examination demonstrated that IL-6 secretion from CAFs was high without stimulation; IL-6 secretion from NFs was markedly lower. IL-6 secretion from CAFs and NFs increased markedly following LPS stimulation. EPA significantly reduced the IL-6-induced increase in secretion in CAFs and NFs (Fig 2A and B). As presented in Fig 2C and D, LPS (50 μg/ml) also increased the VEGF secretion from the two types of human colon fibroblasts. CAFs and NFs secreted higher levels of IL-6 (CAFs, 476.5 pg/ml; NFs, 260.7 pg/ml) and VEGF (54.1 and 60.2 pg/ml) with LPS stimulation compared with the control cells that did not have LPS stimulation (IL-6: CAF, 276.3 pg/ml; NF, 15.2 pg/ml; VEGF: CAF, 19.5 pg/ml; NF, 16.0 pg/ml; Fig. 2A-D). Additionally, EPA (30 μM) significantly suppressed the secretion of IL-6 (CAF, 30.9 pg/ml; NF, 8.2 pg/ml) and VEGF (CAF, 0 pg/ml; NF, 0 pg/ml; all P<0.01) compared with the control group; additionally, EPA (30 μM) significantly suppressed the secretion of both cytokines despite LPS stimulation (IL-6: CAF, 171.4 pg/ml; NF, 57.2 pg/ml; VEGF: CAF, 0 pg/ml; NF, 0 pg/ml; all P<0.001) when compared with the LPS stimulated cells. While EPA suppressed VEGF secretion completely even after LPS stimulation (Fig. 2C and D), it did not completely suppress IL-6 secretion even without LPS stimulation (Fig. 2A and B). On the other hand, MRA, an anti-IL-6 receptor antibody, suppressed the LPS-induced promotion of VEGF secretion. However, the VEGF secretion level from CAFs following LPS stimulation and treatment with 10 μg/ml MRA was not at the same level as that of the control, and no significant difference was identified (Fig. 2E). Whereas MRA significantly suppressed VEGF secretion from NFs to a level that was almost the same as that in the control (P=0.023; Fig. 2F).

Then, to evaluate the association between these secretion levels and inflammation, IL-6 and VEGF secretion levels from CAFs treated with various concentrations of LPS with or without EPA treatment were quantified using ELISA. While LPS promoted IL-6 and VEGF secretion from CAFs in what appeared to be a dose-dependent manner, the addition of 30 μM EPA suppressed these secretions completely, even with increasing LPS concentrations (Fig. 3A and B). Furthermore, the administration of IL-6 to CAFs promoted VEGF secretion in what appeared to be a dose-dependent manner, while the addition of 30 μM EPA suppressed VEGF secretion even with the addition of 10 ng/ml of IL-6 (Fig. 3C).

EPA (30 μM) and 10 μM U0126 reduce p-ERK expression, and IL-6 and VEGF secretion from CAFs. The influence of EPA on VEGF administration in CAFs, in terms of ERK phosphorylation in fibroblast cells, was examined by western blotting (Fig. 4A). ERK phosphorylation, and IL-6 and VEGF secretion from CAFs without EPA treatment were compared with those from CAFs treated with EPA or U0126, a MEK inhibitor. EPA inhibited p-ERK expression in what appeared to be a dose-dependent manner (Fig. 4A). Although no significant difference was identified following 10 μM EPA administration, 30 μM EPA significantly inhibited the p-ERK/GAPDH expression ratio compared with the control group (P=0.0012; Fig. 4B). U0126 also significantly decreased p-ERK expression compared with the control group (P<0.0001). In contrast, EPA

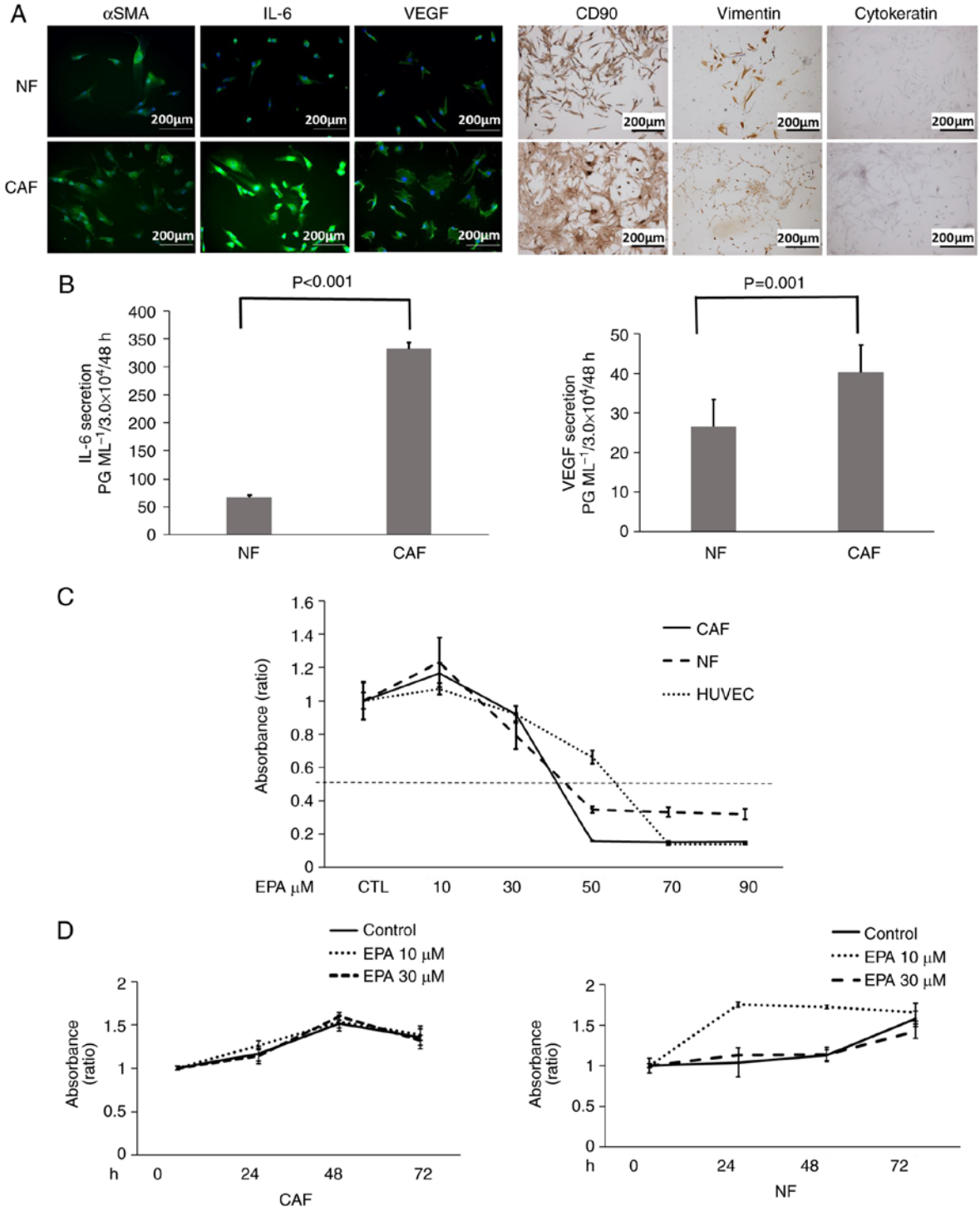

Figure 1. IL-6, VEGF and α-SMA expression is elevated in isolated CAFs, and NFs, CAFs and HUVECs remain viable with 30 μM EPA treatment. (A) Immunofluorescence staining of proteins in NFs and CAFs. (B) Concentration of IL-6 and VEGF secreted in NFs and CAFs, analyzed by ELISA. (C) The WST-1 assay was used to assess cell viability in NFs, CAFs and HUVECs following EPA treatment. The WST-1 assay was used to assess cell viability in (D) CAFs and (D) NFs with and without EPA treatment. Data are representative of three independent experiments and are presented as mean ± standard error of the mean. IL, interleukin; VEGF, vascular endothelial growth factor; SMA, smooth muscle actin; CD, cluster of differentiation; CAF, cancer-associated fibroblast; NF, normal fibroblast; HUVEC, human umbilical vein endothelial cell; EPA, eicosapentaenoic acid.

and U0126 treatment did not significantly affect the expression of total ERK (Fig. 4C). The expression ratio of pERK/ERK was inhibited with U0126 (P=0.0074) and 30 μM EPA (P=0.028; Fig. 4D). Additionally, IL-6 and VEGF secretion from CAFs was significantly inhibited by EPA and U0126 compared with the control group (both P<0.002; Fig. 4E and F).

EPA reduces CSF in HUVECs cultured in CM and serum-free DMEM via the inhibition of VEGF secretion. The angiogenesis assay on Matrigel used to study CSF by HUVECs (Fig. 5) reflects angiogenesis *in vivo*. CSF by HUVECs was observed in the control group (Fig. 5A). EPA suppressed CSF in HUVECs cultured in serum-free DMEM (Fig. 5B). VEGF, which is an

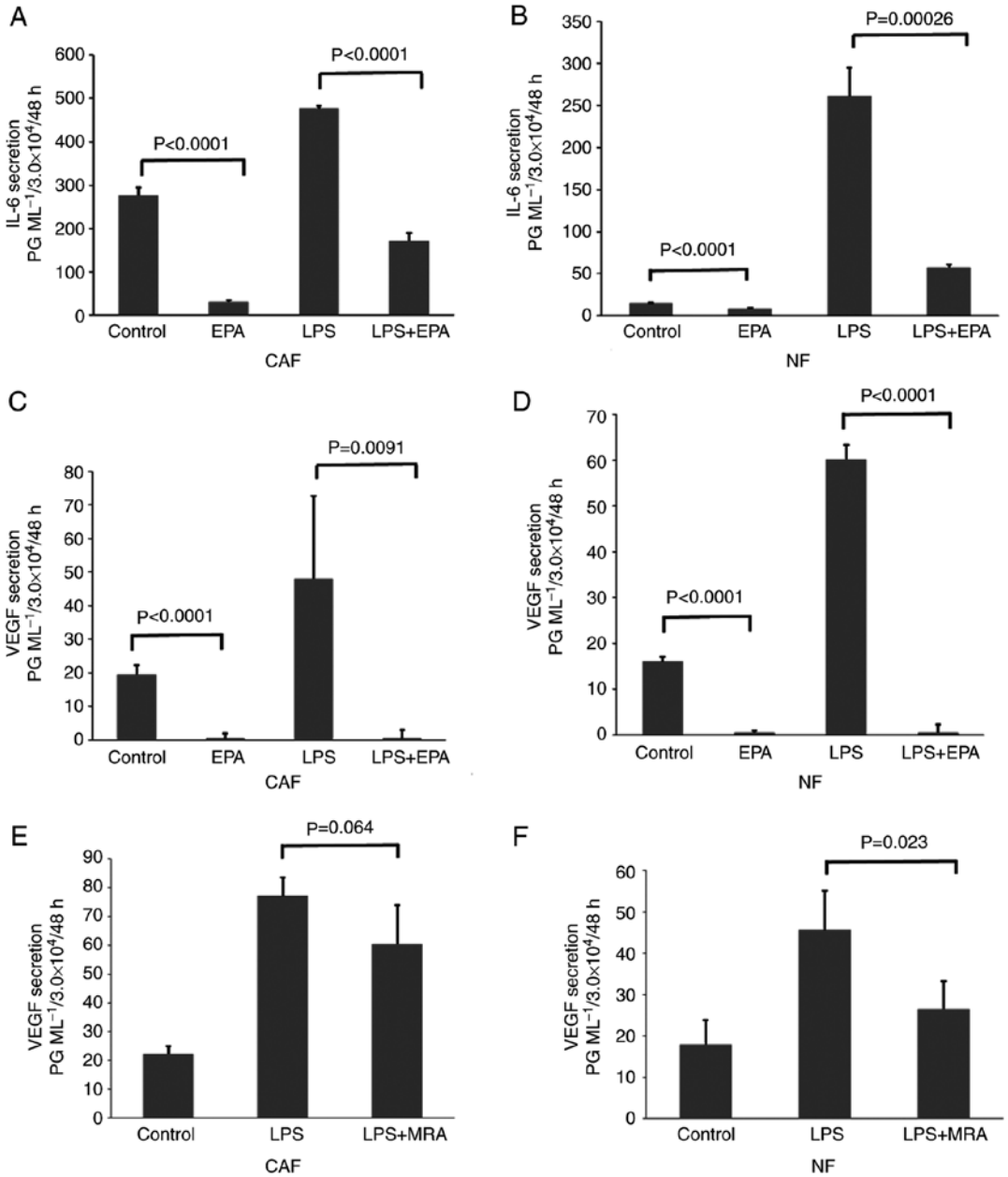

Figure 2. EPA reduces VEGF and IL-6 secretion in NFs and CAFs with or with LPS-stimulation. IL-6 and VEGF protein concentrations were detected by ELISA. (A-D) CAFs and NFs were either untreated, or treated with 30 μM EPA, 50 μg/ml LPS or LPS and EPA. The concentration of IL-6 secreted from (A) CAFs and (B) NFs. The concentration of VEGF secreted from (C) CAFs and (D) NFs. (E and F) CAFs and NFs were either untreated, or treated with 50 μg/ml LPS or LPS and 10 μg/ml MRA. The concentration of VEGF secreted from (E) CAFs and (F) NFs. Data are representative of three independent experiments and are presented as mean ± standard error of the mean. IL, interleukin; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; CAF, cancer-associated fibroblast; NF, normal fibroblast; EPA, eicosapentaenoic acid; MRA, anti-IL-6 myeloma receptor antibodies.

angiogenic factor (26), markedly promoted CSF in HUVECs at a concentration of 500 pg/ml (Fig. 5C). The supernatant obtained from a CAF culture also markedly promoted CSF in HUVECs (Fig. 5D). EPA also suppressed CSF in HUVECs cultured with the CAF supernatant (Fig. 5E). To evaluate whether this CSF suppression by EPA is caused by its direct effect on HUVECs or by VEGF suppression, 500 pg/ml VEGF was added to the HUVECs cultured in the CAF supernatant with EPA (Fig. 5F). The count of tube formation was evaluated in Fig. 5G. The results demonstrated that EPA and VEGF significantly increased CSF in HUVECs compared with the control cells, and that EPA, and EPA and VEGF combined significantly increased CSF in HUVECs treated with CM. These results indicated that CSF decreased due to EPA administration may be caused by the inhibition of VEGF secretion and not by the direct inhibition of HUVEC growth.

EPA decreases VEGF secretion from CAFs by suppressing ERK phosphorylation. From the results in Figs. 1-5, it is clarified that EPA suppresses the secretion of IL-6 and VEGF from CAFs, which causes angiogenesis in cancer (Fig 6). Previous studies revealed that the anticancer mechanisms of EPA are as follows: i) Reduction of IL-6 secretion from cancer cells causes the suppression of tumor proliferation and invasion (11,16,27),

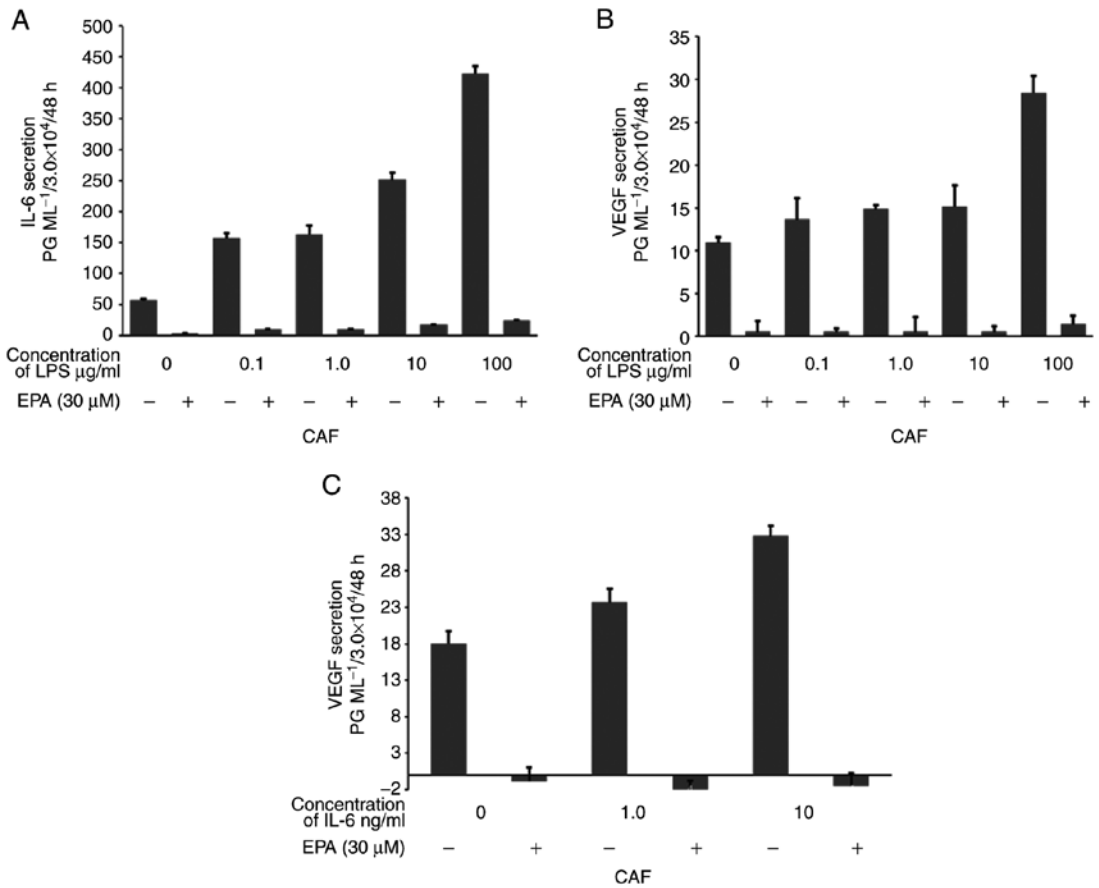

Figure 3. EPA reduces LPS-induced increases IL-6 and VEGF secretion, and IL-6-induced VEGF secretion in CAFs. IL-6 and VEGF protein concentrations were detected by ELISA. (A and B) CAFs were either untreated or treated with various concentrations of LPS with and without 30 μM EPA. The concentration of (A) IL-6 and (B) VEGF secreted from CAFs. (C) CAFs were either untreated or treated with various concentrations of IL-6 with and without 30 μM EPA. The concentration of VEGF secreted from CAFs. Data are representative of three independent experiments and are presented as mean ± standard error of the mean. IL, interleukin; VEGF, vascular endothelial growth factor; LPS, lipopolysaccharide; CAF, cancer-associated fibroblast; EPA, eicosapentaenoic acid.

which may be caused by the suppression of ERK phosphorylation in cancer cells (14,20,28,29), and ii) anti-angiogenesis effects are caused by the direct suppression of endothelial cell proliferation mediated by cyclooxygenases (30). The results of the present study demonstrated novel anti-angiogenesis mechanisms of EPA, involving the decrease of VEGF secretion from CAFs by the suppression of ERK phosphorylation.

CAFs from other colorectal cancer patients remain viable with 30 μM EPA treatment, and EPA reduces VEGF and IL-6 secretion in CAFs from other colorectal cancer patients with or without LPS-stimulation. Examinations of CAFs from other colorectal cancer patients were also performed (Fig. S1). Patient 1 was a 42-year-old Japanese male patient with well-differentiated colon cancer. Patient 2 was a 74-year-old Japanese male patient with poorly-differentiated colon cancer. No significant differences in the cell viability of all fibroblasts were identified in patients treated with or without 30 μM EPA (Fig. S1A and B). LPS (50 μg/ml) stimulation increased IL-6 secretion from NFs and CAFs retrieved from the patients, and EPA (30 μM) suppressed the secretion of IL-6 whether LPS stimulation occurred or not (Fig. S1C and D). LPS also increased VEGF secretion from CAFs, while EPA suppressed the secretion of VEGF in CAFs with and without LPS stimulation (Fig. S1E and F).

Discussion

The present study has revealed a great deal of the mechanism behind the anticancer effects of EPA. One effect of EPA is anti-inflammatory via the suppression of IL-6 secretion. This may be caused by the inhibition of the ERK signal by EPA. IL-6 causes various types of tumor progression, including tumor invasion and tumor proliferation (1). Greten *et al* (31) and Grivennikov *et al* (32) reported that IL-6 also causes tumor angiogenesis via nuclear factor NF-κB. Furthermore, IL-6 has been demonstrated to cause systemic metabolism, which causes cachexia in patients with advanced cancer (13). Thus, it was hypothesized that the inhibition of IL-6 secretion by EPA suppresses tumor progression via several mechanisms (33). For a long time, it was been hypothesized that this mechanism is affected by IL-6 secreted from cancer cells (11). However, a previous study by our group demonstrated that more IL-6 is released from the cancer stroma compared with cancer cells (10). Thus, it remains necessary to clarify whether EPA decreases IL-6 secretion from cancer stromal cells to a level that is same as that from cancer cells.

Another anticancer effect of EPA is its anti-angiogenic effect, which occurs via the direct suppression of endothelial cell growth and tubular formation (30). Szymczak *et al* (30) reported that EPA modulates angiogenesis via cyclooxygenases.

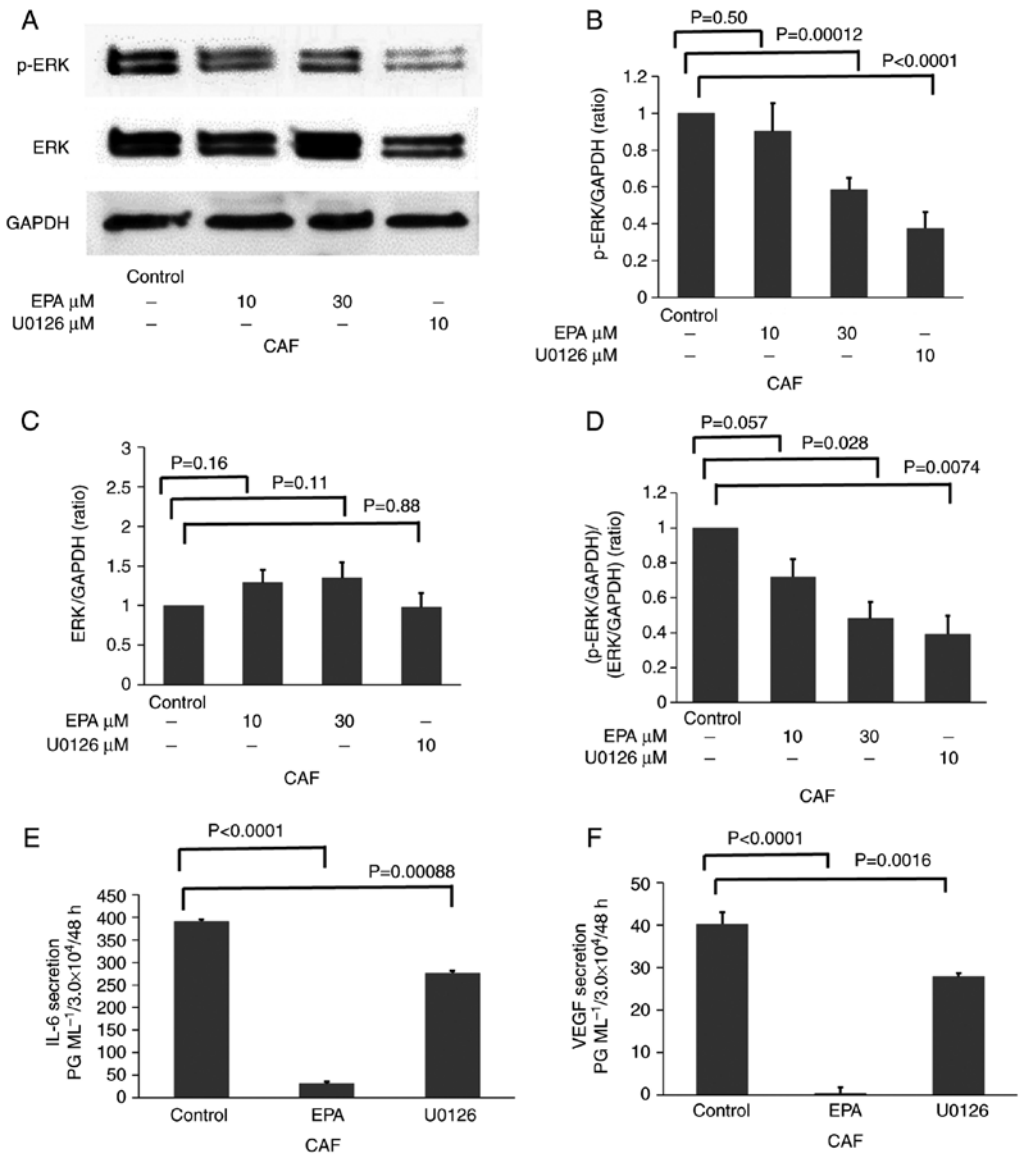

Figure 4. EPA (30 μM) and 10 μM U0126 reduce p-ERK expression, and IL-6 and VEGF secretion from CAFs. CAFs were either untreated, or treated with 10 μM EPA, 30 μM EPA or 10 μM U0126, a dual specificity mitogen-activated protein kinase kinase 1/ERK inhibitor. (A) ERK and p-ERK signals were evaluated by western blotting. The densitometric analysis of (B) p-ERK, (C) ERK and (D) p-ERK/ERK as ratios of GAPDH. The concentration of (E) IL-6 and (F) VEGF secreted from CAFs was analyzed by ELISA. Data are representative of three independent experiments and are presented as mean ± standard error of the mean. ERK, mitogen-activated protein kinase; p-, phosphorylated; IL, interleukin; VEGF, vascular endothelial growth factor; CAF, cancer-associated fibroblast; EPA, eicosapentaenoic acid.

The results of the present study demonstrated that EPA addition suppressed CSF in HUVECs *in vitro*. The authors of the present study hypothesize that this is mechanism may also cause an anticancer effect.

In the present study, the authors demonstrated a novel anticancer mechanism of EPA, which involves an anti-angiogenic effect associated with CSI caused by CAFs and cancer cells. This is caused by the EPA-mediated suppression of VEGF secretion from CAFs. CAFs secreted more IL-6 compared with NFs. Furthermore, LPS stimulation increased IL-6 secretion from CAFs. Additionally, IL-6 increased VEGF secretion from CAFs in what appeared to be a dose-dependent manner. This indicates that inflammation caused by bacterial infection or cancer itself may promote IL-6 secretion from CAFs, which may promote the autocrine secretion of VEGF from CAFs. In contrast, the present study determined that EPA entirely decreased VEGF secretion from CAFs.

Nevertheless, from the results of the present study, two hypotheses remain for the decrease of VEGF secretion from CAFs. One involves the inhibition of IL-6 secretion from CAFs by EPA, which results in the suppression of VEGF secretion from CAFs, and the other states that EPA suppresses VEGF secretion from CAFs directly. EPA suppressed VEGF secretion from CAFs completely despite the increasing levels of IL-6. The results of the present study do not support the theory that EPA decreases the VEGF secretion from CAFs indirectly by decreasing IL-6 secretion from CAFs. The authors of the present study concluded that EPA affects the VEGF secretion mechanism of CAFs directly by inhibiting ERK phosphorylation. The western blotting examination of the present study revealed that ERK phosphorylation reduced

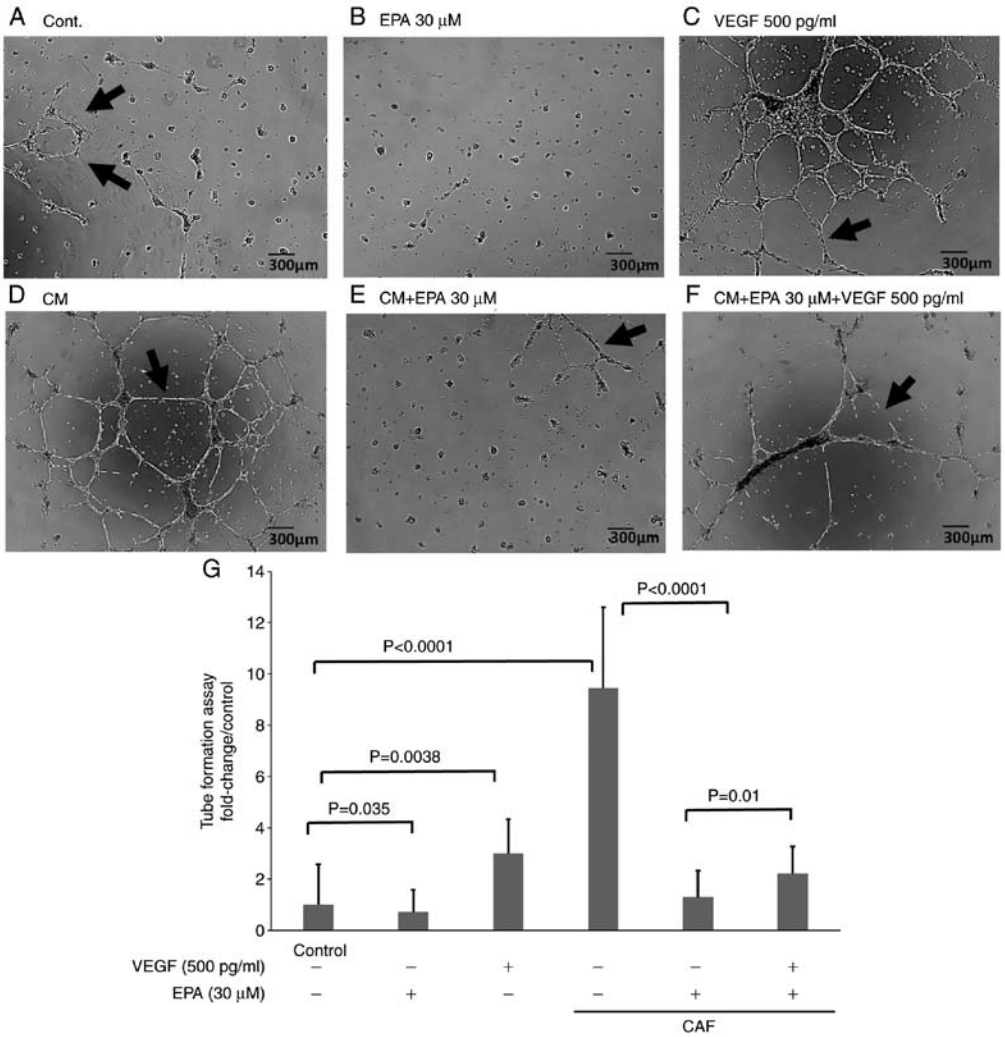

Figure 5. EPA reduces CSF in HUVECs cultured in CM and serum-free DMEM via the inhibition of VEGF secretion. The angiogenesis assay on Matrigel was conducted on HUVECs. CSF (arrow) was counted in nine fields. HUVECs were cultured in (A) serum-free DMEM alone, (B) serum-free DMEM with 30 μM EPA, (C) serum-free DMEM with 500 pg/ml VEGF, (D) CM alone, (E) CM with 30 μM EPA, and (F) CM with 30 μM EPA and 500 pg/ml VEGF. (G) Quantification of CSF. Data are representative of three independent experiments and are presented as mean ± standard error of the mean. HUVEC, human umbilical vein endothelial cell; CSF, capillary-like structure formation; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; CM, supernatant from cancer-associated fibroblasts; EPA, eicosapentaenoic acid.

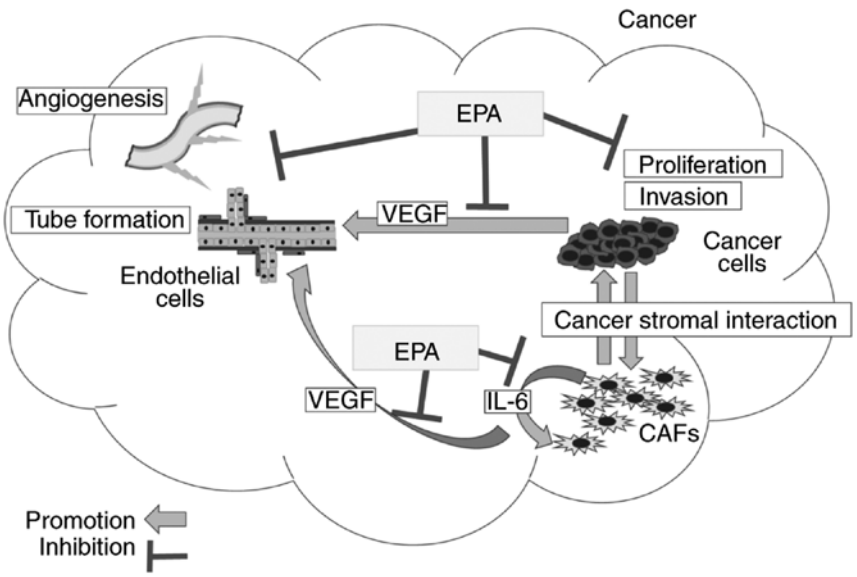

Figure 6. Diagram of the anticancer effects of EPA. VEGF, vascular endothelial growth factor; IL, interleukin; CAF, cancer-associated fibroblast; EPA, eicosapentaenoic acid.

in CAFs treated with EPA or U0126, a MEK/ERK inhibitor. It was also revealed that CAFs treated with EPA or U0126 exhibited decreased IL-6 and VEGF secretion. These results demonstrated that the mechanism elucidated in the present study is similar to the mechanism revealed in another study, which demonstrated that EPA decreased IL-6 secretion from cancer cells (15). It was reported that, in colon cancer cells, ERK activation is a key step in the upregulation of VEGF induced by serum starvation (34). In addition, it was also reported that EPA was able to markedly inhibit ERK-1 and -2 phosphorylation (14,20,28,29).

The origin of CAFs has been discussed for a long time. Several studies demonstrated that insisted stromal fibroblasts transform into CAFs (1,35); however, adipocytes, epithelial cells (through epithelial-mesenchymal transition), endothelial cells (through endothelial-mesenchymal transition), bone marrow-derived mesenchymal stem cells and hematopoietic stem cells are also considered as origins of CAFs (1,36-38). The present study revealed that similar fibroblasts obtained from different regions of the colon had different characters. The present study demonstrated that while it was necessary to stimulate fibroblasts from the normal colon region to secrete IL-6, those from the cancer colon secreted IL-6 without any stimulation. Furthermore, the present study revealed that inhibiting IL-6 could not suppress VEGF secretion completely in CAFs, contrary to the case in NFs. One possible reason for this is that there are many cytokines other than IL-6 released from CAFs without any stimulation (1); these may also influence VEGF secretion. In the present study, the authors succeeded in demonstrating the anti-angiogenic effect of EPA is associated with CAFs; however, there are a number of EPA mechanisms that may explain the suppression of IL-6 secretion from CAFs that remain confounding.

Despite these previous reports that demonstrated the anticancer effects of EPA (11,14-21), it has not been used as an anticancer drug, but just as an anti-coagulant (for example ethyl icosapentate). However, there have not been any studies that investigated cancer stroma, CSI and the utility of EPA in cancer therapy. Cancer tissues consist of cancer cells and stromal cells, and as much as 60-90% of the mass of colon cancers is composed of stromal cells, including fibroblasts, vascular endothelial cells and immune cells (1,39). Actually, stromal fibroblasts are a major component of tumors (40); they have been termed CAFs and they have specific effects on tumor growth (22,41,42). Thus, inhibiting CSI will serve a more important role in cancer therapy compared with solely inhibiting cancer cell activity. The present study demonstrated the effects of EPA on stromal cells in terms of IL-6 and VEGF secretion. From these results, VEGF suppression seems to cause many anticancer effects other than anti-angiogenic effects.

CAFs have not been developed as a cell line for widespread use. Thus, a great number of studies have used cells cultured in the laboratory (43,44). While this may not appear to give reproducible results, the authors of the present study confirmed that the same results were obtained using cells derived from other cancer patients. Thus, this method is universal.

In conclusion, the present study determined that EPA inhibited angiogenesis caused by CAFs and CSI. Thus, EPA could be important for obtaining a variety of anticancer effects in cancer therapies.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

NA, NN and KT performed most of the experiments. TY, YuM and TH collected the cultured fibroblasts. MH, KS and ST designed the study. NA, MH, HI, YoM and HT analyzed the obtained data. ST conducted the entire study. TY, YuM, TH, MH and KS contributed to the conception and design of the study and were also responsible for the acquisition, analysis and interpretation of the data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by Nagoya City University Graduate School of Medical Sciences and Nagoya City University Hospital Institutional Review Board (18-127). Written informed consent was obtained from the patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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